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Cystine Growth Inhibition Through Molecular Mimicry: a New Paradigm for the Prevention of Crystal Diseases

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Abstract

Cystinuria is a genetic disease marked by recurrent kidney stone formation, usually at a young age. It frequently leads to chronic kidney disease. Treatment options for cystinuria have been limited despite comprehensive understanding of its genetic pathophysiology. Currently available therapies suffer from either poor clinical adherence to the regimen or potentially serious adverse effects. Recently, we employed atomic force microscopy (AFM) to identify L-cystine dimethylester (CDME) as an effective molecular imposter of L-cystine, capable of inhibiting crystal growth in vitro. More recently, we demonstrated CDME's efficacy in inhibiting L-cystine crystal growth in vivo utilizing a murine model of cystinuria. The application of AFM to discover inhibitors of crystal growth through structural mimicry suggests a novel approach to preventing and treating crystal diseases.

Keywords

Kidney stones; Cystinuria; L-cystine; Atomic force microscopy; Molecular imposter; L-cystine dimethylester; L-cystine methylester

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Conflict of Interest Michael H. Lee declares no conflict of interest.

Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Introduction

Cystinuria is a genetic disease that clinically manifests as cystine renal stone formation. More than 80 % of people with cystinuria will form their first kidney stone in the first three decades of life [1]. In fact, cystinuria is responsible for 1–2 % of all cases of nephrolithiasis but accounts for up to 8–10% of renal stones in the pediatric population [2–4]. Cystine stones, composed of hexagonal L-cystine crystals, tend to recur and frequently cause chronic kidney disease in people with cystinuria [5, 6]. Three quarters of cystine stone formers will develop stones in both kidneys [7].

The first report of cystine stones dates back to 1810, but it was more than a century later when the pathophysiology of cystinuria was first elucidated [8, 9]. Defective reabsorption from the tubule lumen of filtered cystine by the apical cell membranes of the renal proximal tubule results in accumulation of L-cystine in the urine, predisposing to subsequent crystallization and stone formation [10–13]. In the intestine, there is also a defect of the apical membrane absorptive process. This physiologic shortcoming is compensated for by another apical membrane transporter, solute carrier family 15 member 1 (PEPT1), which allows absorption of cystine and dibasic amino acids, as components of short peptides, from the gut lumen [14]. The presence of PEPT1 in the intestine and its absence in the kidneys explain the urinary hyperexcretion of cystine in cystinuria without associated malnutrition. The defective transport process in the kidney also leads to increased urinary excretion of three dibasic amino acids: lysine, arginine, and ornithine. Although their urinary concentrations are concurrently increased in cystinuria, they are quite soluble at the typical urinary pH of 5–7 and do not contribute to nephrolithiasis [9].

Mutations in two genes underlie the genetic basis of cystinuria: *SLC3A1* on chromosome 2p encodes the neutral and basic amino acid transport protein (rBAT), and *SLC7A9* on chromosome 19q encodes b^(0,+)-type amino acid transporter 1 (b^{0,+}AT) [1, 15]. Both of these proteins are subunits of the transport system b^{0,+} responsible for reabsorption and absorption of cystine and dibasic amino acids at the apical membranes of the proximal renal tubule and small intestine, respectively. rBAT is responsible for trafficking the catalytic, transporting subunit, b^{0,+}AT, to the apical membrane. Defective production of either leads to the phenotype of cystinuria. Mutations in *SLC3A1* produce a disorder with autosomal recessive heritability; heterozygotes have normal urinary cystine excretion. Mutations in *SLC7A9* produce an autosomal dominant disorder with variable penetrance; heterozygotes have abnormal urinary cystine excretion constituting enough to sometimes form stones. About 6 % of people homozygous for mutations in either *SLC3A1* or *SLC7A9* do not form renal stones [16]. Knockout mouse models for both of these genes have been developed and utilized for research on cystinuria [17–19].

Current Medical Treatments of Cystinuria

Although much of the genetics and pathophysiology of cystinuria have been elucidated, no treatment has been developed to improve the defective resorption of cystine and other dibasic amino acids in the proximal renal tubule [15]. Currently, available treatments instead focus on reducing urinary cystine concentration, increasing cystine solubility by raising

urinary pH, or chemically reducing cystine into more soluble compounds. One method of lowering urinary cystine concentration is reducing total sodium intake [20–22]. The positive independent correlation between urinary sodium excretion and cystine concentration has been demonstrated in a study utilizing urinary cystine capacity [23]. The mechanism behind the association between urinary sodium and cystine excretions, however, remains unknown. Another means of decreasing cystine concentration in urine is reducing animal protein intake, which has two effects: it increases cystine solubility by raising urine pH and lowers total methionine (a cystine precursor) and cystine consumption [15, 24]. While these two interventions are commonly recommended to patients, it must be noted that they have not been tested as effective reducers of stone recurrence rates in a robust trial [15]. Urine cystine can be also diluted by increasing fluid intake with extra focus on night-time fluid ingestion to prevent nocturnal crystal aggregation [25]. Titration of fluid intake to 3–4 L of daily urine output is recommended, but even motivated patients may find this difficult to achieve due to social and occupational barriers [26].

Alkalinization of urine can prevent cystine stone development by increasing the solubility of cystine, thereby preventing crystallization and aggregation of cystine molecules. Typical solubility of cystine ranges from 1 to 2 mmol/L, and it increases with a rise in pH [27]. Unlike constituents of other disease-causing crystals, however, the relationship between pH and cystine solubility does not follow a predictable pattern and therefore cannot be described by a nomogram [28]. The lack of straightforward correlation between pH and cystine solubility limits a calculation of cystine supersaturation, and this fact is overcome by directly measuring cystine capacity, or the degree of cystine supersaturation, via a solid-phase assay [29]. In clinical practice, an increase in urine pH can be achieved with oral potassium citrate or sodium bicarbonate [30]. Of the two, potassium citrate is the preferred therapy as it does not result in an undesired increase in cystine excretion, which happens with sodium bicarbonate secondary to increased sodium intake. Citrate also prevents calcium phosphate precipitation which may rarely occur as the result of alkalinization of urine [15]. As previously mentioned, a decrease in animal protein intake also results in an increase in urine pH.

Cystine-binding thiol drugs (CBTDs) can be used for patients who experience recurrent stone formation despite the above measures. Two currently available such drugs are D-penicillamine and α -mercaptopropionylglycine (tiopronin). Cystine is a homodimer of two cysteine molecules $\text{HSCH}_2\text{CH}(\text{NH}_2)\text{COOH}$ connected by a disulfide bond: $\text{COOH}(\text{NH}_2)\text{CHCH}_2\text{S-SCH}_2\text{CH}(\text{NH}_2)\text{COOH}$. CBTDs work by chemically reducing the disulfide bond of cystine and forming drug-bound cysteine complexes, which are up to 50 times more soluble than cystine [31]. The potential for CBTDs to dissolve cystine molecules was demonstrated in a study of seven patients whose urine had higher cystine capacity while taking CBTDs [27]. Although tiopronin may be slightly better tolerated than D-penicillamine, both can cause serious adverse effects, such as rash and proteinuria, not uncommonly making the medications intolerable [32].

Potential New Treatments

The 1980s was marked by the invention of two piezoelectric-based investigational tools, which allow topographical studies of electrode samples at the atomic scale. Scanning tunneling microscopy (STM) utilizes a small metal tip that is brought very close to the sample surface where the distance between the tip and the sample is less than a nanometer [33–35]. Electron flow from the metal tip to the sample electrode generates tunneling current. As the metal tip scans over the sample of interest, a real-space surface image of the sample is generated based on either changes in the current magnitude or variations in the metal tip position along the vertical axis. This method allows refined three-dimensional contour mapping of the sample surface while achieving unprecedented resolutions up to 0.1 Å (angstrom= 10^{-10} m) [36]. Soon after its development, STM was shown to be applicable to studies of biologic samples in addition to metals and semiconductors [37].

A similar application of piezoelectrics to the microscopic topographical study is atomic force microscopy (AFM), which combines the principles behind STM and stylus profilometry [38]. Instead of utilizing tunneling current, AFM measures the force of interaction between the metal tip, or the cantilever, and the sample of interest [34]. A laser beam aimed at the cantilever tip deflects to a photodiode detector, and as the cantilever is brought near or in contact with the sample surface, force between the tip and the sample causes the cantilever to bend. This transient cantilever deformation changes the position of the laser on the photodiode, and a collective recording of these changes provides useful information about the sample surface.

AFM has revolutionized the study of crystal diseases by allowing measurement of adhesion forces between additive molecules and crystal surfaces [39, 40]. AFM can quantify “ultrasmall” forces in the order of 10^{-18} N, smaller than the interactive force between individual atoms [38]. For example, AFM cantilevers modified and coated with urinary proteins and functional biological groups, such as carboxylate and amidinium moieties, have been used to study aggregation and epithelial adhesion of calcium oxalate urinary stones [41, 42]. By using cantilevers of known spring constants, differences in the amount of force required to detach the modified cantilevers from calcium oxalate crystal surfaces are measured, which, in turn, provide information regarding urothelial microenvironments favoring or impeding calcium oxalate stone growth.

Another major advantage of AFM is its ability to provide real-time in situ visualization of growing crystals [43]. Through AFM, growth of the hexagonal cystine crystals in a left-handed spiral pattern has been demonstrated in supersaturated (2 mM) L-cystine solution (Fig. 1) [44•]. Attachment of individual L-cystine molecules to the six equivalent {100} faces of the hexagon results in spiral enlargement of the hillock into a larger hexagonal plate. This unique function of AFM has recently led to a new paradigm in designing novel treatments for crystal diseases, in particular cystinuria, by allowing identification of a molecular imposter against crystal growth.

Molecular imposters are inhibitor compounds that hinder crystal growth through their structural resemblance to the original crystal constituent molecules [45]. Binding moieties of

these imposters allow them to attach to crystal surfaces, but their perturbing moieties obstruct additional solute molecules from binding to their neighboring sites, thus deterring further crystal enlargement. The role of various molecular imposters has been explored in many areas, including naturally occurring antifreeze proteins and calcium oxalate renal stones [46–48].

The ability to visualize crystal growth in real time with AFM has been utilized to study effects of molecular imposters on cystine crystals [44•]. L-cystine dimethylester (CDME), a structural mimic of L-cystine with the carboxylate groups substituted by bulkier esterified methyl groups [$\text{CH}_3\text{O-COCH}(\text{NH}_2)\text{CH}_2\text{S-SCH}_2\text{CH}(\text{NH}_2)\text{CO-OCH}_3$], was shown to be an effective inhibitor of cystine crystal growth. CDME effectively lowered the growth rate of individual hexagonal cystine crystals in supersaturated L-cystine solution, leading to a drastic decrease in crystal dimensions. CDME inhibited cystine crystal growth at concentrations as low as 2 mg/L (<0.01 mM), and 5 mg/L of CDME resulted in a thousand-fold reduction in cystine crystal size. CDME also distorted the hexagonal architecture of the crystal. The role of CDME as a molecular imposter illustrated by AFM was confirmed in the macroscopic level; CDME significantly decreased total mass yield in bulk crystallization. Interestingly, L-cystine methylester (CME), with only one carboxylate group replaced by a methylester group, similarly inhibited cystine crystal growth, but the magnitude of changes in the crystal growth parameters was smaller than those seen with CDME [44•]. This observation supports the role of CDME and CME as molecular inhibitors of crystal growth where the methylester groups function as perturbing moieties.

The efficacy of CDME in cystine crystal growth suppression was recently demonstrated in vivo using a *Slc3a1* knockout male mouse model, further supporting molecular mimicry as an area of potential answers to cystinuria and other crystal diseases [49•]. Feeding 10 mg/kg of CDME daily for 4 weeks resulted in a significant decrease in stone size and total stone burden despite a concomitant increase in the number of cystine stones (Fig. 2). CDME decreased the average weight of individual cystine stones by more than sevenfold (from 7.0 to 1.1 mg). This observation is in agreement with the in vitro finding that CDME simply blocks crystal growth without participating in L-cystine metabolism [44•]. Even at high doses of CDME (5 mg/mL in water supply for 4 weeks equivalent to 20–25 mg CDME daily), no apparent adverse effects were observed, and the kidneys, bladder, and liver were without evidence of pathological changes in the post-mortem examination [49•]. Although previous murine studies of cystinosis, a lysosomal storage disease, showed mixed results regarding potential toxicity conferred by CDME, these prior experiments differ from our study in that CDME was parenterally administered [50–54]. A toxic effect of enteral CDME consumption has not been reported to date.

One potential problem with animal studies and future clinical trials may be hydrolysis and de-esterification of CDME by the abundant esterases present in the blood, intestine, and liver, which can diminish the effective urinary concentration of CDME. In order to overcome this potential limitation, our group has designed new cystine crystal growth inhibitors, which are resistant to esterases (Dr. Longqin Hu, personal communication). These new compounds are highly hydrophilic in nature and could potentially be absorbed

through luminal transporters of the small intestine. Effectiveness of these compounds is currently being studied.

The AFM-assisted discovery of CDME as a potential growth inhibitor of cystine crystals and the subsequent confirmation of its effect in vivo with the murine model represent a major potential shift in the paradigm of managing patients with crystal diseases. For cystinuria, this molecular mimic of L-cystine suggests a new treatment strategy that does not rely on changing the urine microenvironment via increased fluid intake or alkalization. Although an adverse effect profile of CDME in humans has not been delineated, it may no longer be necessary to chemically reduce L-cystine with thiol drugs at the expense of their burdensome side effects. More importantly, this strategy of utilizing molecular mimicry is easily translatable to other crystal diseases. Unexplored potential areas of new treatment development include monosodium urate crystallization in gout and prevention of acyclovir-induced crystalluria. Identification of effective imposters with AFM will select new candidate molecules to be tested in animal models.

Conclusion

The application of AFM to cystinuria has led to the successful identification of the effective molecular imposter CDME. The potential use of CDME in the clinical setting awaits a clinical trial demonstrating its efficacy and determination of its adverse effect profile. This novel approach to developing new therapies for cystinuria can be translated to other types of pathogenic crystals and suggests a new paradigm for the prevention of crystal diseases.

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Fig. 1.

a, b Real-time in situ AFM images of a *L*-cystine crystal, acquired 12 min apart. A pair of hexagonal hillocks generated by two closely spaced dislocations serves as landmarks. **c, d** AFM images of a single dislocation center of *c* *L*-cystine and *d* *D*-cystine crystal during growth. **e, f** AFM image of a hexagonal growth hillock on the (001) face of *L*-cystine before and after the addition of *L*-CDME (5 mg/L; 0.02 mM), revealing roughening of the {100} steps due to step pinning. Images were acquired in aqueous solutions containing 2 mM *L*-cystine. Reproduced with permission from *Science*

Fig. 2.

Bladder stones recovered from two water-fed (*left*) and three CDME-fed (*right*, 10 mg/mL daily for 4 weeks) knockout male mice. The stones recovered from CDME-fed mice are more numerous in number and smaller in size compared to those of water-fed mice